Quantitation and Reproducibility Problems in Reversed-Phase and Size-Exclusion HPLC Analyses of Wheat Proteins[1]

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ABSTRACT

Reversed-phase (RP-) and size-exclusion (SE-) high-performance liquid chromatography have become important methods for rapid identification of wheat and other cereal cultivars and for revealing quality differences. Accuracy and reproducibility are essential for good results. Due to recent changes in these methods, however, such as using smaller columns, lower flow rates, and smaller samples, small procedural errors become more critical for final results. We therefore further studied the causes and magnitude of problems involving quantitation and reproducibility in RP- and SE-HPLC analyses of wheat proteins. Because of potential problems with protein adsorption to stainless steel system components, we modified systems to contain, insofar as possible, all plastic-type components. Other potentially major problems included weighing flour samples, achieving accurate solvent composition, and interpretation of data. Recognizing and dealing with these problems will enhance the accuracy, reproducibility, and usefulness of HPLC for varietal identification and quality prediction.

Fractionation of cereal proteins by high-performance liquid chromatography is still a relatively young method. The first practical separations of wheat and maize proteins by reversed-phase (RP-) and size-exclusion (SE-) HPLC were reported in 1983–1984 (Bietz 1983, 1984). Improvements in columns and techniques came rapidly, as noted in the first book devoted to HPLC of cereal and legume proteins (Kruger and Bietz 1994). Because of its improved reproducibility and speed, and the ability to accurately quantify results, especially as compared to gel electrophoresis, HPLC quickly became one of the most important methods used by many researchers to analyze cereal proteins.

In spite of these advances, some problems remain with these methods. For example, during our analysis of a large sample set for which quantitative data were of utmost importance, breakdowns necessitated reanalyzing many samples. The second sets of results often differed significantly from the first. Analytical SE- and ion-exchange HPLC confirmed this reproducibility problem. We also became aware of a study (Dolan 1996) that showed that proteins could adsorb to HPLC injector seals or other system components, affecting quantitative precision. We have thus further investigated the causes of this irreproducibility. We here show that various factors (many of which have not previously been well recognized) can cause problems in quantitative results from HPLC of cereal proteins.

METHODS

Samples

Wheat flour samples were from numerous hard red spring and winter wheats grown in 1988 and collected by Federal Grain Inspection Service personnel at grain elevators during unloading. Hard wheats were grown in areas typical for their classes and were milled at the USDA-ARS Wheat Quality Laboratory, Fargo, ND. Soft red winter wheats were from the USDA-ARS Soft Wheat Quality Laboratory, Wooster, OH, or were obtained directly from breeders at universities where varieties had been developed. Breeders' wheat kernels were pulverized with a Wig-L-Bug (Huebner et al 1990), and sieved to remove bran; only flour passing through a 500-μm screen was analyzed.

Protein Extraction

Flour (60 or 70 mg) was extracted for 30 min with vortex shaking at room temperature with 1.5 mL of 70% ethanol in 10-mL polypropylene tubes. Extracted gliadins were clarified by centrifugation (9,000 × g for 10 min at 25°C) before analysis. To isolate glutenins, gliadin extraction was first performed twice (with 0.9 and 0.6 mL 70% ethanol), and extracts were combined. Glutenins were then solubilized with 1.5 mL of 0.05M sodium phosphate (pH 7.7) + 0.1% dithiothreitol, containing either 5.5*M* urea or else a combination of 3.5*M* urea + 2*M* guanidine hydrochloride (GuHCl). After 2 hr of vortex shaking, samples were centrifuged (12,000 × *g* for 10 min) and placed into autosampler vials, and 5 μL of 33% 4-vinyl pyridine in 60% propanol (10–30% more than needed to alkylate all reduced protein sulfhydryl groups plus the remaining dithiothreitol) was added. The mixture was then vortexed gently for 1 hr, after which 15 μL of a 60:40 mixture of trifluoroacetic acid (TFA) and acetic acid was added to lower the pH to 3.0 ± 0.2.

SE-HPLC

Gliadin and reduced-alkylated glutenin samples were analyzed by SE-HPLC on a 1- × 30-cm Superose-12 column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) with an inline 2-μm polyetheretherketone (PEEK) filter (A-429X, Upchurch Scientific, Oak Harbour, WA). The solvent system was modified slightly from that used previously (Huebner et al 1994, 1997) to prevent degradation of column material at low pH. For gliadins, the solvent used was 44% acetonitrile + 0.08% TFA + 35 m*M* NaCl, adjusted to pH 2.95 ± 0.05 with NaOH. For glutenins, the solvent was 38% acetonitrile + 0.12% TFA + 1*M* urea + 35 m*M* NaCl, adjusted to pH 2.95 ± 0.05 with NaOH. Sample size was 10 μL; flow rate was 0.55 mL/min; and column temperature was 34°C. All samples were analyzed at least in duplicate, and often three or more times. Proteins were detected at 210 nm (10 mV) with a Spectroflow-100 UV monitor (ThermoQuest/Thermo Separation Products, Schaumburg, IL).

RP-HPLC

Gliadin and reduced glutenin samples were analyzed by RP-HPLC with a 2.1- × 150-mm Vydac C_{18} column protected with a 4- × 12.5-mm guard column (MAC-MOD Analytical, Chadds Ford, PA), using a P4000 solvent delivery system and an AS3000 autosampler (ThermoQuest/Thermo Separation Products). Columns were maintained at 60°C; flow rates were generally 0.3 mL/min; and sample size was generally 4.5 μL.

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Data Analyses

RP- and SE-HPLC data, expressed as voltage output of the UV monitor, were stored in a personal computer and integrated with PC-1000 software (ThermoQuest/Thermo Separation Products).

RESULTS

Enzymes may adsorb to stainless steel tubing and be destroyed during HPLC analysis. Some recent HPLC systems therefore replace stainless steel tubing, columns, and filter materials with titanium or PEEK. Since we do not routinely analyze enzymes, and since initial RP-HPLC results showed good reproducibility and recovery (Bietz 1983, Bietz et al 1984), we had largely ignored the possibility of quantitation error due to poor recovery of some proteins. While analyzing a large group of samples that required accurate quantitation, however, we noticed occasional significant quantitative differences and irreproducibility upon repeat analyses. This problem was confirmed when these samples were analyzed on another column type and with a different HPLC system. Subsequent studies examining reproducibility revealed several potential problems.

Stainless Steel

As noted above, we occasionally experienced unexplained problems with reproducibility. Today, HPLC systems increasingly use inert (e.g., PEEK) tubing and other materials where system parts contact samples. We therefore modified one dedicated RP-HPLC system so that all stainless steel tubing and other parts that normally contact samples (except for the column) were replaced with inert (mainly PEEK) materials. Initial results indicated a large increase in gliadin recovery using PEEK tubing. It was later determined, however, that these increased recoveries also represented integration problems due to baseline irregularities. Similar results were noted for reduced-alkylated glutenins. These experiments are not easily repeatable, however, because of difficulties in reconfiguring HPLC systems. In another older system that could be more easily reconfigured, major increased recoveries using PEEK tubing were not observed. Nevertheless, the variable recoveries observed when comparing stainless steel with more inert materials, plus the quantitative differences often observed between initial and replicate RP-HPLC separations within a series, strongly suggest that some wheat proteins adsorb to stainless steel system components and that this can affect quantitative reproducibility. The extent of such adsorption may decrease after adsorptive sites become saturated with protein.

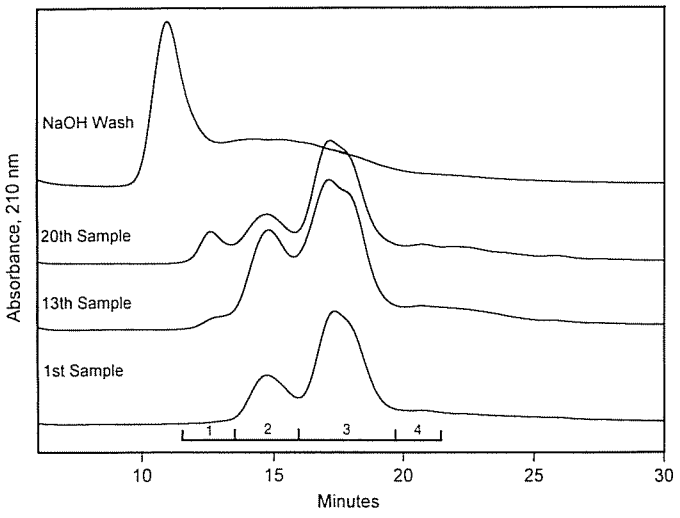

Fig. 1. Repetitive size-exclusion HPLC analyses of reduced-alkylated glutenin. Results for the 1st, 13th, and 20th injections are shown. After 30–40 injections, 10 µL of 1*N* NaOH (a volume equal to that of the sample) was injected (top pattern). Fractions 1–4 are, respectively, highly aggregated or unreduced glutenin, high molecular weight glutenin subunits, low molecular weight glutenin subunits, and albumins and globulins.

SE-HPLC

SE-HPLC is often used to relate wheat protein molecular size distribution to functional characteristics. Baseline stability during such analyses generally assures accurate quantitation. However, another problem may exist. Figure 1 shows replicate SE-HPLC analyses of a reduced-alkylated glutenin sample. After several runs, a peak that was initially absent began to appear at ≈12–13 min. After 30–40 analyses, injection of 10 µL of 1*N* NaOH released a significant amount of early-eluting high molecular weight (HMW) material that had apparently adsorbed to the apparatus or at the inlet to the column packing; a second NaOH injection produced no peak.

It is conceivable that such adsorptive phenomena may be less severe using different solvent conditions or columns. It is evident, however, that wheat protein adsorption can easily occur when typical SE-HPLC conditions are used.

We also noted, from increases in pressure, that the polypropylene filters typically present at the column inlet and outlet became plugged with time. Washing with dilute NaOH and other strong solvents did not clean these filters sufficiently to permit their continued use. Filters typically had to be removed and replaced after 60–80 analyses of reduced-alkylated glutenin; many more gliadin samples could be analyzed before the filter became plugged. Replacing polypropylene filters with Whatman no. 54 paper filters eliminated this problem; possibly other filter material types, such as PEEK if available, would also be suitable. After the polypropylene filters were replaced, results became reproducible after two to three analyses, and at least a few hundred samples could be analyzed before the filters had to be replaced. It is somewhat difficult, however, to prepare and properly insert paper filters of an exact size into an HPLC column without disturbing its packing.

Solvents

To explain the absence of the HMW glutenin SE-HPLC peak noted initially during a series of analyses (Fig. 1), many solvent systems and reagents for reduction of glutenin were tested. Changing the denaturant in the reducing solution from 5.5*M* urea to 3.5*M* urea + 2*M* GuHCl increased recovery of reduced and alkylated glutenin (Fig. 2). For minor fractions such as fraction 1, the relative difference was very great. The increased ionic strength of the urea + GuHCl solvent probably prevented samples from adhering to stainless steel tubing or other system materials. It is possible, but not likely, that this urea + GuHCl solvent simply extracts more protein, since more material is also eluted if GuHCl is added to a 5.5*M* urea extract before SE-HPLC (results not shown). Before anal-

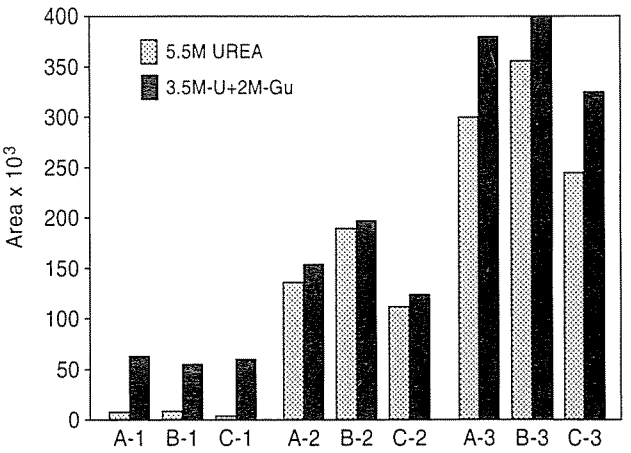

Fig. 2. Size-exclusion HPLC recoveries for glutenin reduced and alkylated in the presence of either 5.5*M* urea or 3.5*M* urea + 2*M* guanidine-HCl. A–C designate three samples, and 1–3 designate three fractions (Fig. 1).

ysis, samples have also typically been passed through a 0.45-μm Millipore filter in a stainless steel holder, providing another opportunity for protein adsorption, especially for small samples. We therefore now use an all-plastic filter holder; with this change, addition of GuHCl to solvent or samples may be less necessary.

As noted previously, glutenin subunits, even after reduction and alkylation, may aggregate and precipitate with time, resulting in quantitative differences (Fig. 3). Thus, it is usually best to analyze samples immediately, but this is not always possible. Samples that are reanalyzed also obviously differ in age, and glutenin subunit types differ significantly in tendencies to precipitate with time (Fig. 3). The apparent amount of fraction 1 decreases most with time. Fraction 1 consists primarily of low molecular weight (LMW) polypeptides that characteristically aggregate and elute at the void volume upon size-exclusion chromatography (Huebner and Wall 1974). These polypeptides are very different from major HMW and LMW glutenin subunits (Huebner et al 1974) and require a strong disaggregating solvent to be separated from other glutenin subunits (Huebner and Wall 1980). Fraction 2 (Fig. 3) corresponds to glutenin's major HMW subunits. Of glutenin's major subunit types, these have an intermediate tendency to precipitate with time; they may be separated from LMW glutenin subunits by, for example, neutralization and cryoprecipitation (Bietz and Wall 1973). Fraction 3 (Fig. 3) consists of glutenin's LMW ethanol-soluble subunits, which are least likely to precipitate. Nevertheless, even after 10 days, brief sonication with a sonic cleaning device, combined with gentle agitation and heat, resolubilized at least part of all precipitated glutenin subunit fractions (Fig. 3).

Sample Preparation

Finely milled wheat flour can adhere to many materials by electrostatic attraction (a reviewer of this article rightly noted that static in plastic tubes can be significantly reduced by using an antistatic gun, such as the Milty ZeroStat, commonly used to reduce static on audio recordings) and can also be easily suspended in gently moving air. Such factors can make accurate weighing of wheat flour samples into small containers, such as 10-mL centrifuge tubes, especially difficult. Figure 4 compares results of a study in which the same amount of flour was weighed either on weighing paper or directly into tubes. Direct weighing into tubes is generally preferred since recoveries are 2–4% higher and a transfer step is avoided.

In a second study, two persons weighed a set of 10 samples for RP-HPLC analyses. Gliadin recoveries were identical for four samples and varied from 3.0 to 4.6% for five samples—but one sample varied by 36% (data not shown); such a large difference could also be due to other unidentified problems.

In a third study, three persons having similar laboratory skills weighed the same set of flour samples using weighing paper (Table I). The resulting quantitative differences for comparable samples were sometimes surprisingly large, illustrating the typical quantitative experimental variation that can be introduced by this procedure. Total gliadin recoveries for the 12 samples weighed by the three individuals averaged 100, 99.3, and 97.6%. Variation for individual samples was considerably higher (e.g., 6.1–7.7%) and did not always follow the same pattern among weighers. These results emphasize the necessity of replicate analyses in quantitative studies and suggest that use of the largest recovery values—representing minimum loss of material during weighing and transfer—may be preferable to the use of averages, especially when one result is much lower than another.

Other Miscellaneous Problems

In addition to the problems described above, other errors may occur. Many of these were reviewed by Marchylo (1994). For example, sample volume must be minimal in RP-HPLC so that all solutes adsorb to the column packing (Marchylo and Kruger 1988). We have also noted that a quantitative variation of more than 2–3% for duplicate analyses of the same sample usually indicates an HPLC system problem, such as air bubbles in the syringe. Air bubbles can be caused by a restricted sample delivery tube, by the needle, or by too-rapid syringe movements. Even with helium-degassed solvents, after one to two days, dissolved air may again be present and must again be removed by helium degassing or vacuum. Small, invisible bubbles in sample delivery tubing must also be flushed out with freshly degassed solvent. Other possible system problems that could influence quantitative accuracy include gradient blending or solvent delivery uncertainties.

Sample viscosity may also be a problem with some autosamplers. If the syringe rate is too fast, viscous liquids may not pass through small openings quickly enough, causing volumetric errors. This might be prevented by, for instance, a brief (1–3 sec) delay after sample injection. Also, injector seals must be replaced regularly to prevent leakage and maintain accuracy.

Extraction problems can also greatly influence reproducibility. Accurate measurement of solvents and proper mixing of flour with solvent during extraction must occur to achieve optimal quantitative accuracy of HPLC results. For example, during extraction, flour should be continuously suspended by vortex or reciprocal motion, at a constant speed, and for a specified time. This is especially critical in sequential extractions, since centrifugation can pack residues so tightly that they are not easily and fully redispersed by casual vortexing (even for 30 min) during subsequent extractions.

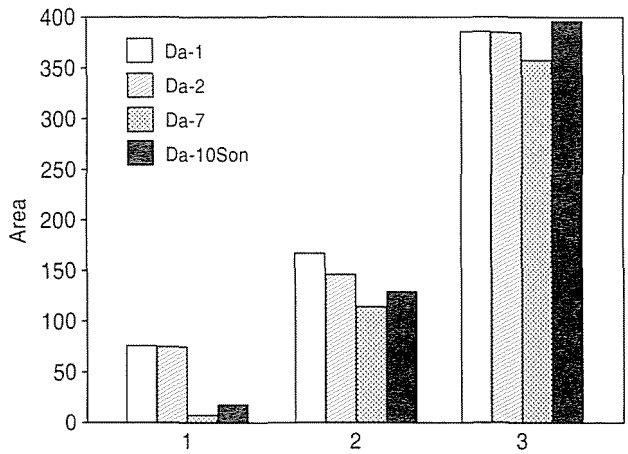

Fig. 3. Recovery by size-exclusion HPLC of glutenin subunit fractions 1–3 (as in Fig. 1), analyzed one, two, and seven days after their preparation, and after 10 days following brief sonication.

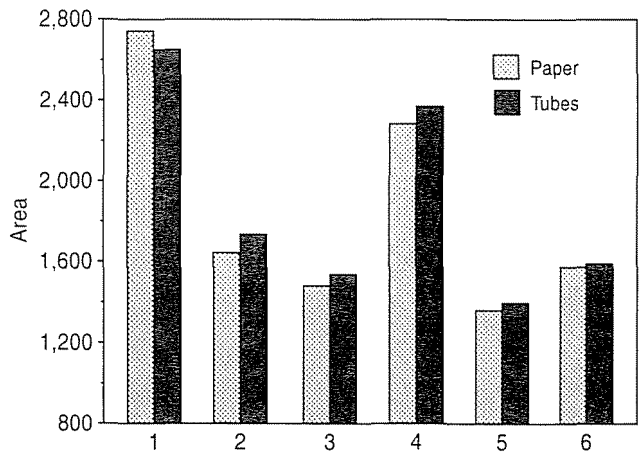

Fig. 4. Total recovery, estimated by size-exclusion HPLC, of gliadins from six wheat flour samples either weighed (by the same person) on weighing paper and then transferred to tubes or weighed directly into tubes.

TABLE I
Reversed-Phase HPLC Recoveries of Gliadins from 70-mg Flour Samples Weighed by Three Persons[a]

Sample	Weigher 1	Weigher 2	Weigher 3	Sample with Lowest % Recovered
A	4842	4606	4615	2 - 95.1
B	4448	4495	4310	3 - 95.9
C	5105	5192	5193	1 - 98.3
D	4363	4590	4510	1 - 95.1
E	3755	3820	3740	3 - 98.0
F	3760	3848	3860	1 - 97.5
G	2959	2958	2905	3 - 98.2
H	4455	4495	4220	3 - 93.9
I	3670	3505	3390	3 - 92.3
J	4564	4690	4528	3 - 96.5
K	2710	2730	2600	3 - 95.2
L	4080	4125	3980	3 - 96.5
Total % recovered	99.3	100.0	97.6	

[a] Data reported as peak area $\times 10^{-3}$.

Inaccurate placement of baselines during integration can also cause huge quantitative variation in HPLC studies. This is especially true in RP- and ion-exchange HPLC gradient procedures. For example, one sample set was analyzed on subsequent days with minor gradient variations. On one day, recoveries were consistently lower. Careful scrutiny of the data showed that the blank run for that day was higher than normal, apparently because the system was not fully equilibrated at the start of analyses. Since the integration method automatically subtracted this baseline from each sample, low apparent recoveries resulted. This anomaly emphasizes that an HPLC system must be at a stable equilibrium at the start of a set of analyses. It is also a reminder of the necessity of replicate analyses (especially early in a sample set) and of the need to visually inspect raw HPLC data carefully and thoroughly and not blindly accept integrated values.

DISCUSSION

We have noted and considered several problems that can commonly occur during quantitative HPLC analyses. Many of these problems are self evident—but they can be easy to overlook. For example, new personnel may use slightly different procedures or may not appreciate the unique difficulties associated with analysis of wheat flour proteins. Results from different equipment types may also vary significantly. Recognition of these difficulties plus constant vigilance are necessary for optimal quantitative results.

We might have presented more detailed statistical data relating quantitative results to various causes, but we chose instead to discuss, in more general terms, the typical range and extent of variation experienced in many studies. In general, these same problems can exist and the caveats will apply in all laboratories, although, obviously, differences in equipment and procedures will affect the amounts of variation associated with any cause.

Of the potential problems in achieving accurate quantitation, perhaps the most troublesome is weighing of samples. Inaccurate sample size also makes it especially difficult to recognize problems resulting from factors such as adsorption, solubility, or equipment problems, which may further erode the accuracy and reliability of quantitative results.

Our results also emphasize the necessity of careful and frequent use of standards during HPLC analyses. While it would seem most desirable to use commercially available purified protein standards, often the best standard for HPLC of wheat proteins is a similar heterogeneous protein mixture, freshly prepared from a stable bulk sample (e.g., a frozen flour) by a standard extraction procedure and analyzed under carefully defined conditions (Bietz 1986). Changes in elution time, resolution, or recovery reflect problems with methodology or equipment that could lead to false information.

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